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Immortalization and characterization of human peritoneal mesothelial cells

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The mesothelial lining of the peritoneal cavity represents a permeability and host defense barrier against local infection [1, 2]. During peritoneal dialysis the mesothelial monolayer is exposed to high concentrations of glucose, repeated changes in pH and osmolality and to inadvertently inoculated microorganisms that may cause inflammation and peritonitis [3]. Inflammatory changes can eventually result in ultrafiltration failure limiting the efficacy of peritoneal dialysis [3]. The study of interactions between the peritoneal dialysates, mesothelial cells (MsC) and inflammatory cells requires stable and differentiated MsC in culture. Permanent peritoneal mesothelial cells are not widely available and primary MsC cultures must be used for *in vitro* studies. The slow growth of primary MsC, their special culture requirements and the need to prepare new primary MsC after four to six passages due to dedifferentiation, limits their usefulness [4, 5]. We, therefore, established a permanent peritoneal MsC line through transfection with SV40 T antigen [6–8]. Using a multitude of cell markers and characteristics we show that this human MsC line maintains characteristics of primary MsC and can now serve as a reproducible model of human MsC.

Methods

MsC were obtained from a consenting surgical patient who underwent elective laparotomy for a reason other than malignancy, and were prepared as described elsewhere [4]. Culture medium for primary cells was Dulbecco's modified eagle medium (DMEM; Biochrom KG, Berlin, Germany) supplemented with 10% fetal calf serum (BioWhitaker, Walkersville, USA), 10% human serum (Sigma, Deisenhofen, Germany), penicillin 100 U/ml/streptomycin 100 μg/ml (Biochrom KG), endothelial cell growth factor (Boehringer Mannheim, Mannheim, Germany) 5 μg/ml, and heparin 5 U/ml (Braun, Germany). For selection of stably transfected cells G418 (Sigma) was used. Transfected cells were cultured in DMEM with 2.5% or 10% bovine serum (Serum supreme; BioWhittaker) and penicillin/streptomycin 100 U/100 μg/liter and with additional glucose (final concentration 2000 mg/liter; Braun). Cells were incubated at 37°C in a humidified atmosphere of 5% CO_2.

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For primary MsC culture the dishes (Greiner Labortechnik, Frickenhausen, Germany) and flasks (Primaria, Falcon) had to be coated with a fibronectin stock solution (50 μg/ml; Boehringer Mannheim) diluted in PBS using 1.65 μg/cm^2, to allow adherent growth of the cells. The transfected MsC line can be grown in regular dishes without pretreatment. Medium was changed twice weekly. Confluent cells were split 1:10 or 1:20 (passages 1 to 4)

To immortalize primary MsC the plasmids pUC Inwt and pRc/CMV were used [7]. pUC Inwt was a gift from E. Fanning (Munich, Germany). It contains SV40 sequences in pUC12 with a deletion in the late region extending from the *Bam*HI site (pos.2533) to the *Pst*I site (pos.1988). A 1 bp insertion at the SV40 origin causes a defect in replication. The pRc/CMV confers resistance to the antibiotics neomycin and geneticin (G418). For transfection 10^7 cells were suspended in 400 μg of PBS with the addition of 10 μg of pUC Inwt and 2 μg of pRc/CMV (both linearized through digestion with *Eco*RI) and electroporated (GenePulser; Bio Rad) using 960 mcF and 230V. Cells were allowed to recover for three days, followed by selection of stably transfected cells using G418 at a concentration of 800 μg/ml, which was lethal for primary cells. On day 17, twelve clones were picked. The morphology of the stably transfected cells was regularly examined by phase contrast microscopy and documented after 10 and 52 doublings.

For immunofluorescence, cells were grown in chamber slides (Lab-Tek, Naperville, IL, USA) until approximately 75% confluence was reached, fixed in 1:1 (vol:vol) methanol/acetone, washed with PBS and TWEEN, and then incubated with the respective antibodies. After 60 minutes unbound antibodies were washed off and the cells were incubated for another 60 minutes with 50 μl of an anti-rabbit TRITC conjugated secondary antibody (SV40 T antigen and VWF) or anti-mouse FITC conjugated secondary antibody (both diluted 1:40 in PBS) [9]. Immunofluorescence micrographs were obtained using a microscope (Leica DMRBE, Germany) equipped for photography (Leica DMRD) with Kodak films. For electron microscopy cells were grown on coverslips (LabTec) until confluent and fixed with 2% glutaraldehyde for 60 minutes. Further processing including embedding and staining with 1% tannic acid was performed as described [10].

For the isolation of genomic DNA, confluent cells (clones 2, 3, 4 and 9) were lysed with EDTA/SDS and incubated with RNAse A at 37°C for one hour. The DNA was purified by phenol/chloroform extraction, precipitated with ethanol and redissolved in TE buffer. For Southern blots, genomic MsC DNA was

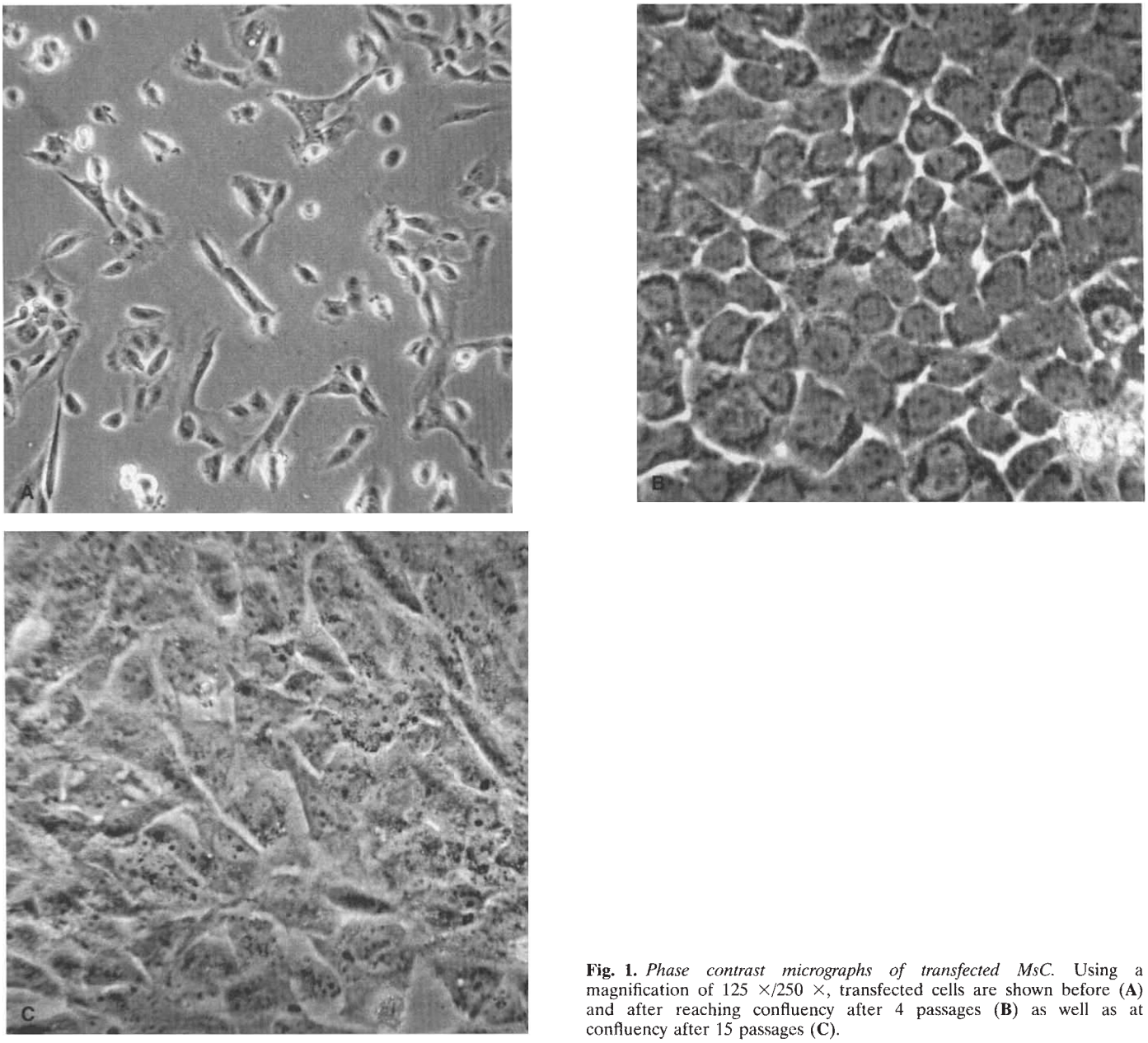


Fig. 1. *Phase contrast micrographs of transfected MsC.* Using a magnification of 125 ×/250 ×, transfected cells are shown before (**A**) and after reaching confluency after 4 passages (**B**) as well as at confluency after 15 passages (**C**).

digested to completion with *Eco*RI, separated on a 0.8% agarose gel, blotted onto a nylon membrane (GeneScreen; NEN, Boston, MA, USA) and hybridized with a radioactively labeled SV40 T antigen probe using standard hybridization conditions [11]. Plasmid dilutions representing 1, 3, 5 and 10 copies of the SV40 T antigen were loaded onto the same gel to serve as the standard. The number of integrated SV40 T antigen copies in MsC was estimated by comparing the signal intensities on the autoradiograph with the standards.

For determination of t-PA and PAI-1, primary and transfected cells were grown to confluency in six-well plates and then stimulated in serum free medium with recombinant human TNF-α (Boehringer Mannheim) at concentrations of 10, 100, 500 and 1000 U/ml. Stimulation with lipopolysacharide (LPS; Sigma) and human interleukin 1α (IL-1; Boehringer Mannheim) was also performed. The concentrations of t-PA and PAI in the supernatant of stimulated and unstimulated cells were determined by ELISA (Coaliza, Chromogenix). For determination of IL-6 release confluent transfected MsC were stimulated with recombinant human TNF-α at concentrations of 100 U/ml, 1000 U/ml and control, and IL-6 concentration in the supernatant was determined by ELISA.

The concentrations of SpA in the supernatant as well as a suspension of homogenized cells was measured with ELISA (Byk Goulden, Konstanz, Germany). Intravital fluorescence microscopy (Leica DMRBE) after exposure to phosphin3R for two minutes at room temperature was performed on transfected cells and primary MsC. To determine the phospholipid composition of

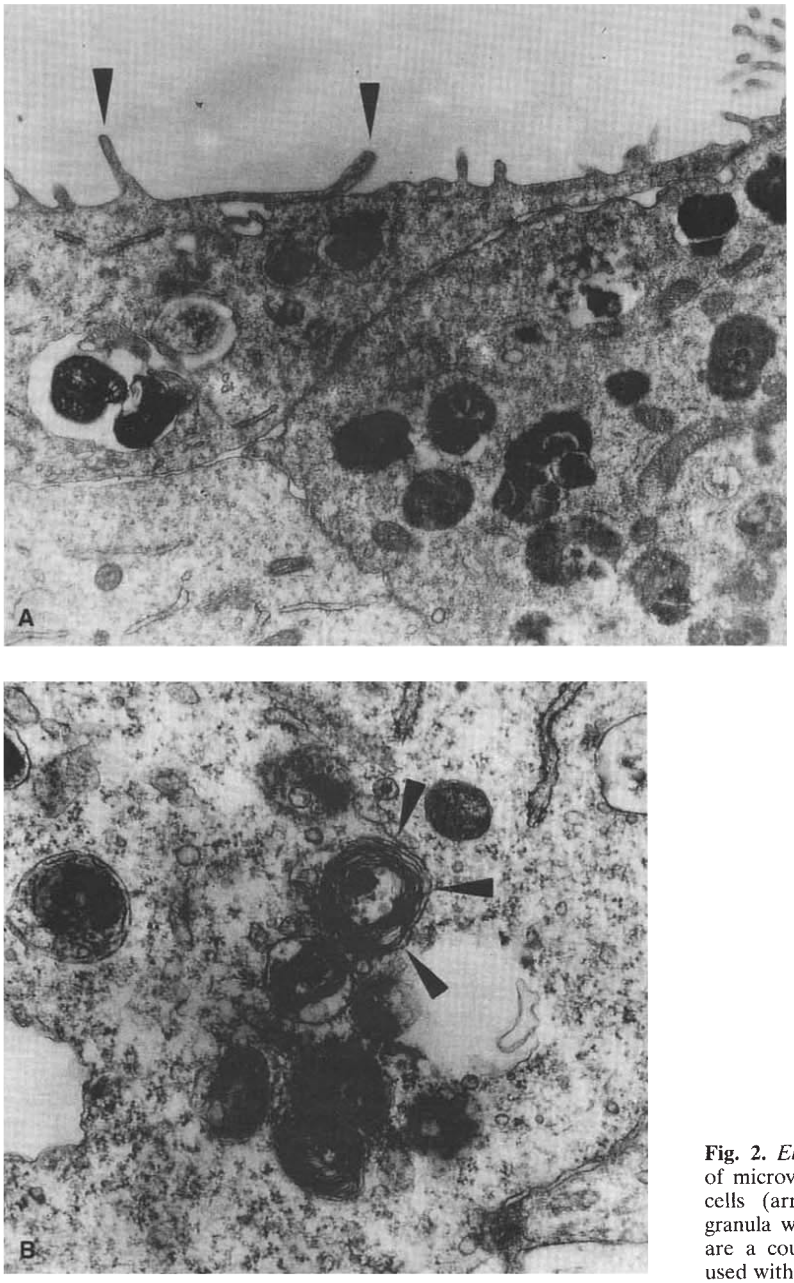


Fig. 2. *Electron micrograph of transfected MsC.* (**A**) Note the presence of microvilli on the cell surface and tight junctions between individual cells (arrow). (**B**) At higher magnification (×11,000) intracellular granula with lamellar appearance can be observed (arrow) (Micrographs are a courtesy of P. Mundel and W. Kriz, University of Heidelberg; used with permission).

the supernatant, cells were washed with PBS twice, incubated for 72 hours with serum-free DMEM ± dexamethasone (Sigma) 1 μM and the supernatant analyzed by standard thin layer chromatography [12].

Expression of WT1 was shown using immunofluorescent staining with a commercially available antibody (C19; Santa Cruz Biotechnology, Heidelberg, Germany) as described above.

Results and Discussion

Nine of the twelve clones could be continuously passaged and showed a pattern of slow growth after three to five passages, with an increase in generation time between the doublings. This phenomenon has previously been described as "crisis," implicating the death of non-immortalized cells with a transient prolongation of their life span. Only clones 4 and 9 could be maintained past the fifth passage post-transfection (approximately 20 doublings), but showed also a sigmoidal growth curve suggestive of a temporarily limited ability to proliferate. Thereafter, stable growth with a generation time of approximately three days per doubling was noted. Re-exposure to 800 μg/ml G418 after 34 doublings did not affect cell growth, proving the persistent integration of the neomycin resistance gene. All subsequent studies were conducted on clones 4 and 9.

The transfected cells require at least 2.5% serum in the medium without additional growth factors or coating of culture dishes. The cells were determined to be free of HIV, hepatitis C, hepatitis B and mycoplasma. Karyotype analysis showed a human hypodiploid karyotype. On Southern blot analysis of various clones a consistently low number (~ 3) of integrated copies of SV40 T antigen per cell was detected.

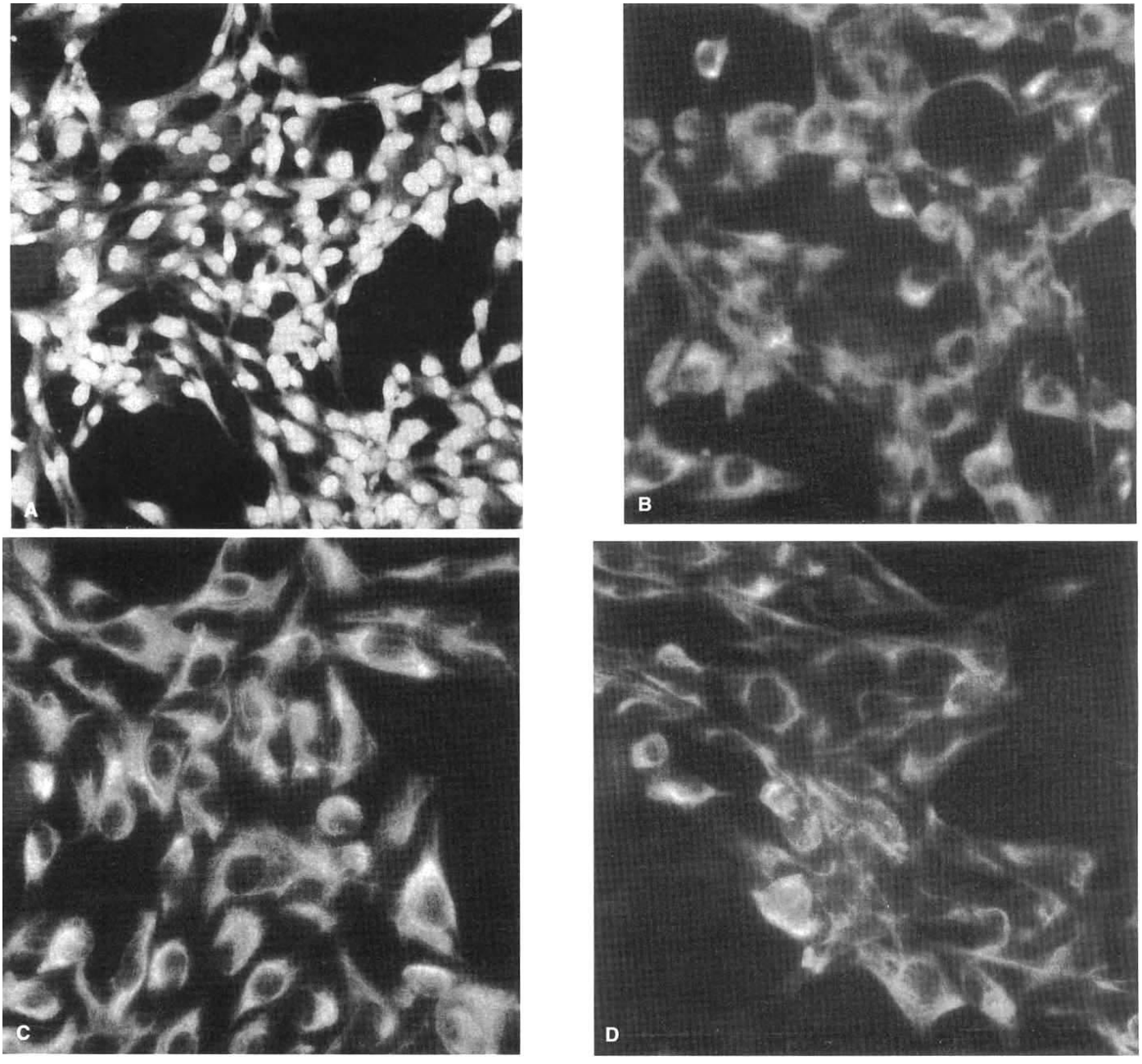


Fig. 3. *Indirect immunofluorescent staining of transfected MsC using antibodies against SV40 T antigen* (**A**), *cytoceratin 8/18* (**B**), *cytokeratin 18* (**C**), *and vimentin* (**D**).

The transfected cells showed no apparent differences relative to primary MsC in morphology during growth, and displayed the typical cobblestone pattern after reaching confluency (Fig. 1).

Figure 2 shows electron micrographs of transfected cells after 6 passages, that is, 24 doublings. Typical for MsC, the monolayer shows tight junctions and polarization with microvilli on the surface [13]. The cytoplasm contains mitochondria, rough endoplasmic reticulum, lipid inclusion bodies and parallel electron dense and electron lucent bands arranged as concentric spheres, highly suggestive of lamellar bodies. All these features are seen in primary mesothelial cells [13]. To further evaluate a mesothelial phenotype immunofluorescent staining was performed (Fig. 3). A comparison and data from the literature are given in Table 1. The presence of cytokeratins 8 and 18 as well as vimentin is a generally

Table 1. Comparison of indirect immunofluorescent staining of primary and transfected peritoneal MsC

Antigen	Primary cells	Transfected cells	Reference
Vimentin	positive	positive	[4]
Cytokeratin 8/18	positive	positive	[5]
Cytokeratin 18	positive	positive	[4]
CD 44	positive	positive	[15]
SV40 T antigen	negative	positive	
PAL-E	negative	negative	[5]
EN 4	negative	negative	[5]
von Willebrand	negative	negative	[6]
control	negative	negative	

After methanol/acetone fixation, primary and transfected cells were stained with the respective antibodies as described in the **Methods** section.

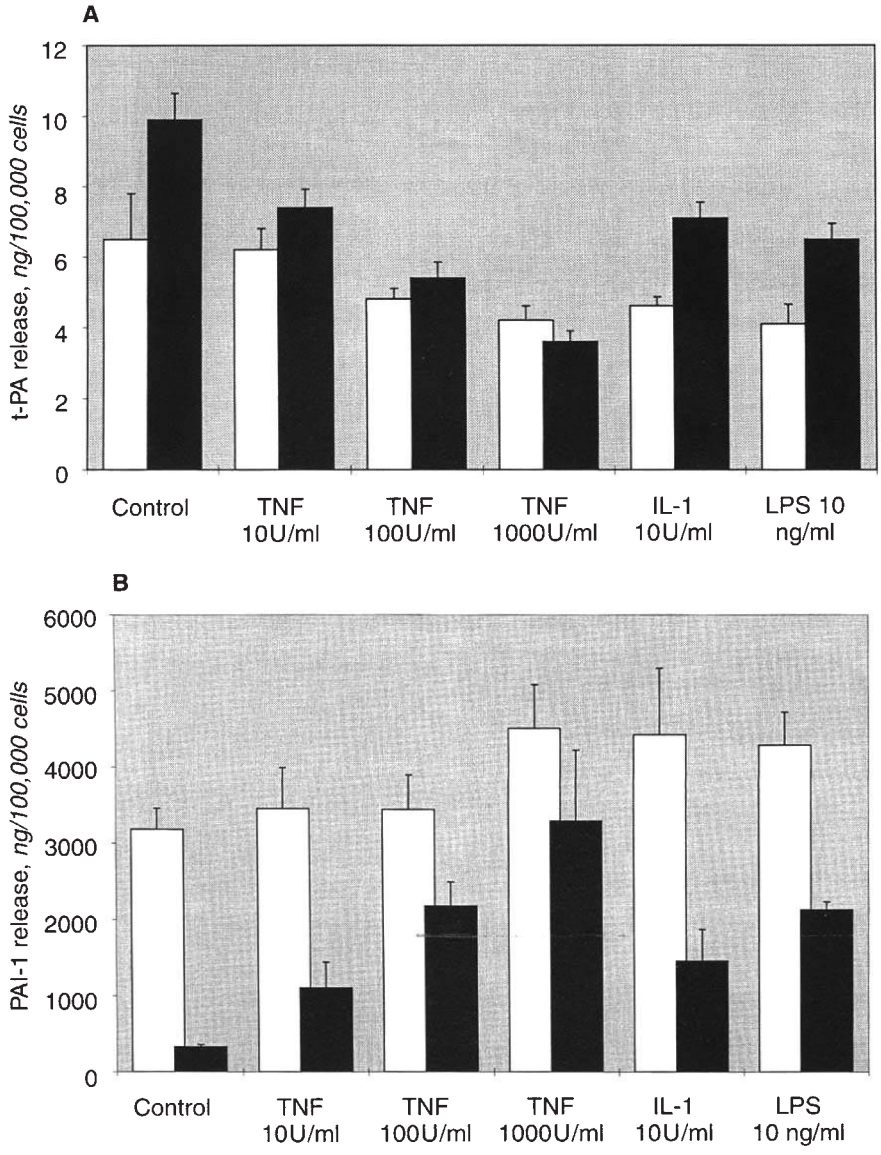


Fig. 4. *(A) Generation of tissue plasminogen activator (t-PA) and (B) plasminogen activator inhibitor (PAI-1) by primary (□) and transfected mesothelial cells (■).* MsC were incubated in serum free medium with lipopolysaccharide (LPS), interleukin 1α and various concentrations of tumor necrosis factor α (TNF) and aliquots analyzed by ELISA. Results are expressed as mean ± SEM of 3 sets of experiments.

accepted feature of mesothelial cells [4, 5, 9, 14]. The absence of endothelial markers strongly argues against endothelial contamination (negative results are not shown). The presence of the SV40 T antigen after 55 doublings, along with the persistent G418 resistance, demonstrates that these cells are stably transfected [7].

Expression of t-PA and PAI has been studied in primary MsC [16, 17]. Figure 4 shows the amount of t-PA and PAI in the supernatant of primary and immortalized MsC. A suppression of t-PA concentrations in the supernatant after TNF-α stimulation is observed for both primary and transfected cells. A similar but less prominent effect occurs after stimulation with LPS or IL-1 (Fig. 4A). In contrast, PAI-1 release into the supernatant can be induced with TNF-α, LPS or IL-1 in primary as well as transfected MsC (Fig. 4B) [16, 17]. These stimuli also cause a dose dependent release of IL-6 into the supernatant (Fig. 5) compared with control, which has also been described in primary MsC [18].

The generation and reuptake of surfactant-like substances were other hallmarks of MsC [13, 19, 20]. In pneumocytes II, phosphin3R is actively transported into the cytoplasm and stored in lamellar granula in the same manner as SpA [21]. Intravital

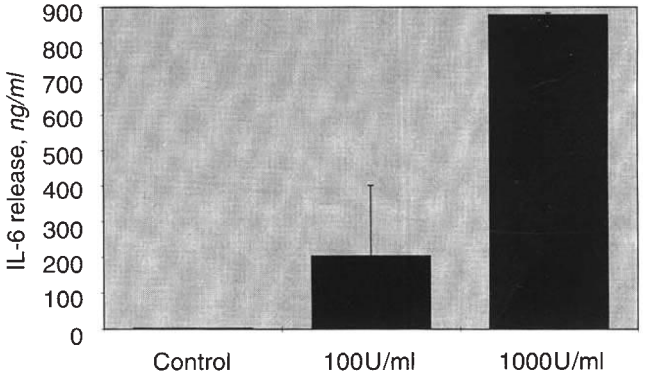
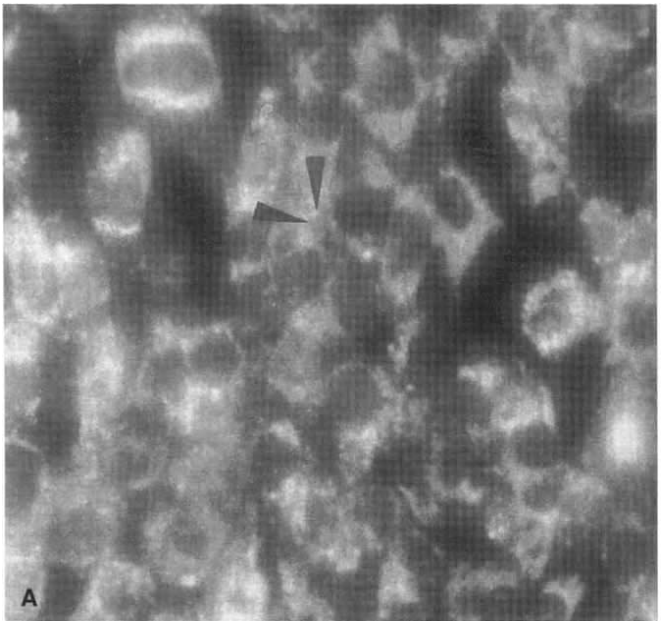


Fig. 5. *Generation of interleukin 6.* Following incubation of transfected MsC with TNF-α the IL-6 content of the supernatant was determined by ELISA. Results are expressed as mean ± SEM of 3 sets of experiments.

fluorescence microscopy of MsC shows a marked uptake of phosphin3R (Fig. 6A) for primary and transfected cells. The cell culture supernatant contained 2.0 ng/100 μl of SpA on ELISA.



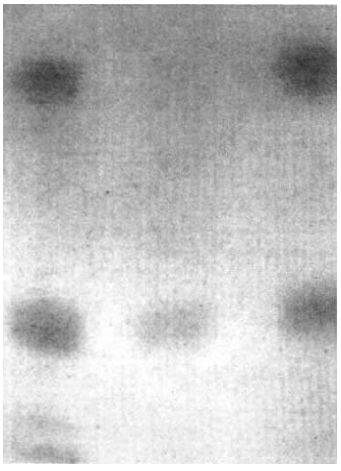


Fig. 6. *Surfactant generation by MsC.* (**A**) Intravital immunofluorescence of transfected MsC with phosphin3R, indicative of surfactant protein A reuptake, demonstrates characteristic green fluorescence (arrow). (**B**) Thin layer chromatography of the culture medium [in regular DMEM (**C**) and with the addition of dexamethasone (**D**)] of transfected MsC shows characteristic bands for phosphatidylcholine, sphingomyelin and kephalin.

The homogenate of MsC showed an intracellular SpA concentration of 57.5 ng/100 μl, similar to that of rat type II pneumocytes [22]. These results demonstrate the presence of surfactant-like material that is also supported by the presence of lamellar bodies on electron microscopy and the results of the phospholipid differentiation (see below). These data are in complete agreement with the presently scant knowledge about production of surfactant like material by primary MsC [13]. The secretion of phosphatidylcholine and sphingomyelin by primary MsC has been shown previously [19, 20]. On thin layer chromatography we were able to show the same components in the supernatant of the transfected MsC line, as further evidence for surfactant production (Fig. 6).

A highly specific marker of mesothelial cells in adults is Wilms tumor gene WT1 [23]. On immunofluorescent staining of transfected MsC a characteristic nuclear pattern can be observed (Fig. 7), again underlining the mesothelial origin of transfected MsC.

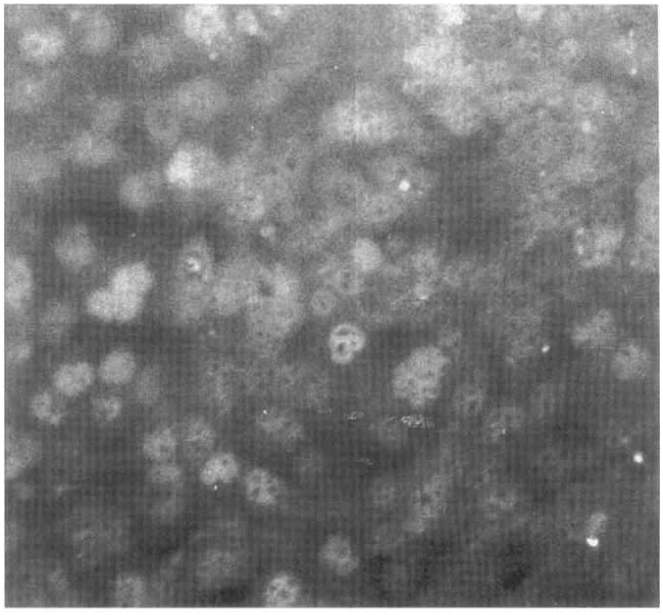


Fig. 7. *Expression of WT1 by MsC: Immunohistochemical staining of transfected MsC using an antibody against WT1 diluted 1:50.*

Conclusion

In conclusion, we have established and broadly characterized a permanent human peritoneal mesothelial cell line that retains the characteristic features of primary MsC. This MsC line will now enable us to perform detailed studies of peritoneal MsC biology in general and of specific influences of various dialysate components.

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Reprint requests to Prof. Dr. Detlef Schlöndorff, Medizinische Poliklinik, Ludwig-Maximilians-Universität, Pettenkoferstr. 8a, D-80336 München, Germany.

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